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A Role for a TIMP-3-Sensitive, Zn^{2+}-Dependent Metalloprotease in Mammalian Gamete Membrane Fusion

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During fertilization, sperm and egg plasma membranes adhere and then fuse by a mechanism that is not well understood. Zinc metalloproteases are necessary for some intercellular fusion events, for instance, cell–cell fusion in yeast. In this study we tested the effects of class-specific and family-specific protease inhibitors on mouse gamete fusion. Capacitated, acrosome-reacted sperm and zona-free eggs were used in assays designed to define the effects of inhibitors on sperm–egg plasma membrane binding or fusion. Inhibitors of the aspartic, cysteine, and serine protease classes had no effect on sperm–egg binding or fusion. Both a synthetic metalloprotease substrate (succinyl-Ala-Ala-Phe-amidomethylcoumarin) and the zinc chelator 1,10-phenanthroline inhibited sperm–egg fusion but did not decrease sperm–egg binding. The fusion-inhibition effect of phenanthroline was reversible and activity of the inhibitable zinc metalloprotease was shown to be required during a short time window, the first 15 min after insemination. Tissue inhibitor of metalloprotease-3 and Ro 31-9790, specific inhibitors of zinc metalloproteases in the matrixin and adamalysin families, also inhibited sperm–egg fusion but not sperm–egg binding. These data indicate a role in gamete fusion for one or more zinc metalloproteases of the matrixin and/or adamalysin families that act after plasma membrane binding and before sperm–egg membrane fusion.

Key Words: **fertilization; sperm–egg fusion; matrixin (MMP); adamalysin (ADAM); zinc metalloprotease.**

INTRODUCTION

Recent research has revealed that protease action regulates the function of key plasma membrane proteins during development (Hooper *et al.*, 1997). For instance, it is required for mouse fetal development that transforming growth factor β (TGF-β) be proteolytically cleaved from its membrane anchor and released as an active, soluble form. The zinc metalloprotease tumor necrosis factor α converting enzyme, TACE (ADAM 17), catalyzes TGF-α cleavage (Peschon *et al.*, 1998). Another example is Notch, a cell surface signaling receptor. Notch signaling regulates cell-fate determination in multiple aspects of vertebrate and invertebrate development. Proteolytic cleavage is necessary for activation of Notch. Notch probably undergoes three different proteolytic cleavages that allow ligand binding and signaling (Lewis, 1998; Schroeter *et al.*, 1998). One of the cleavages that activates Notch may be performed by the zinc metalloprotease kuzbanian (ADAM 10) (Pan and Rubin, 1997; Lewis, 1998). Alternatively, kuzbanian may process to an active form the transmembrane protein Delta, the ligand of Notch (Qi *et al.*, 1999).

The matrix metalloproteases (MMPs) also degrade and modify the extracellular matrix during reproduction and development (Brenner *et al.*, 1989; Matrisian, 1992; McIntush and Smith, 1998). A family of proteins called tissue inhibitors of metalloproteases (TIMPs) is important for the regulation of MMP activity and is thought to be coordinately regulated and expressed with various MMPs (Matrisian, 1992; Apte *et al.*, 1995). One example of this coordinated expression occurs during the implantation of mammalian embryos. Gelatinase B (MMP-9) is primarily responsible for invasion of embryonic trophoblast cells into

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the maternal endometrium, while the level of TIMP-3 produced during implantation is thought to control the degree of trophoblast invasion (Graham and Lala, 1991; Librach *et al.*, 1991; Leco *et al.*, 1996).

Our lab's interest in development is to understand the mechanism of gamete fusion in mammals. Results from yeast fusion studies suggest that cellular fusion events, potentially related to mammalian gamete fusion, involve zinc metalloproteases. Mutational analysis has demonstrated that cell–cell fusion in yeast requires the zinc metalloprotease Axl1p (Elia and Marsh, 1998), an ortholog of the mammalian zinc metalloprotease IDE (insulin-degrading enzyme) (Fujita *et al.*, 1994; Adames *et al.*, 1995).

Metalloproteases have previously been reported to have important roles at two stages during fertilization. Using a heterologous system (human sperm fusing with zona-free hamster eggs), Meizel and colleagues used inhibitors to study an "early" metalloprotease activity. In their studies, gamete fusion was inhibited only if metalloprotease inhibitors were present during induction of the acrosome reaction. Thus the proposed early metalloprotease is posited to act on human sperm during the acrosome reaction. This protease is probably in the acrosomal contents and is presumed to act on the human sperm surface, thereby affecting the sperm's ultimate ability to fuse with zona-free hamster eggs. In these studies, a 50–60% decrease in the number of sperm that fused per egg was obtained by inhibiting the early metalloprotease (Diaz-Perez *et al.*, 1988; Diaz-Perez and Meizel, 1992). Lennarz and colleagues studied a "late" sperm metalloprotease activity that acts after sea urchin sperm have acrosome reacted and bound to the egg plasma membrane. Inhibiting the late metalloprotease resulted in a virtually complete block (>95%) to gamete fusion (Roe *et al.*, 1988).

Our lab and others have worked to establish the identity of gamete surface proteins that may function in mammalian sperm–egg plasma membrane binding. These include the sperm surface proteins fertilin and cyritestin and the egg integrin $\alpha 6\beta 1$ (Primakoff *et al.*, 1987; Myles *et al.*, 1994; Almeida *et al.*, 1995; Evans *et al.*, 1995, 1997, 1998; Linder and Heinlein, 1997; Yuan *et al.*, 1997; Chen *et al.*, 1998; Cho *et al.*, 1998; Chen and Sampson, 1999). Adhesion between sperm and egg plasma membranes may be achieved by interaction of these molecules (and others) and this interaction might lead to or regulate subsequent steps that result in fusion. In this study we asked if, after mouse sperm–egg binding has occurred, there is another step involving zinc metalloprotease action that must occur before membrane fusion.

MATERIALS AND METHODS

Materials and media. Chemicals were purchased from Sigma unless otherwise indicated. The protease inhibitors used were actinonin, captopril, α_2-macroglobulin, 1,10-phenanthroline, phosphoramidon, and Ro 31-9790 (generously provided by Roche Discovery, Welwyn, England); and E64, pepstatin, and Pefabloc SC (purchased from Boehringer Mannheim, Mannheim, Germany). Human TIMP-1 and recombinant human TIMP-3 proteins were a generous gift from Dr. Gillian Murphy (University of East Anglia, Norwich, UK). Synthetic peptides used were *N*-succinyl-alanine-alanine-alanine-7-amido-4-methylcoumarin (succ-Ala-Ala-Ala-AMC) and *N*-succinyl-alanine-alanine-phenylalanine-7-amido-4-methylcoumarin (succ-Ala-Ala-Phe-AMC, a metalloprotease substrate). 4,7-Phenanthroline was purchased from Aldrich Chemical Co.

Media used were M2 and M16 (Hogan *et al.*, 1994), containing 0.3% BSA, and Whittingham's (Whittingham, 1971) containing 3% BSA. Media used for *in vitro* fertilization (IVF) assays were equilibrated under mineral oil overnight at 37°C/5% CO_2 in air. All manipulations of gametes were carried out in equilibrated media under mineral oil, and incubations were at 37°C/5% CO_2, unless indicated otherwise.

Preparation of gametes. Female (6–8 weeks) and male (10–12 weeks) ICR mice were purchased from Charles River Laboratories (Wilmington, MA). Female mice were superovulated as described previously (Yuan *et al.*, 1997). Oviducts were excised from superovulated females, and eggs were released into equilibrated M16 medium without mineral oil. Egg–cumulus complexes were transferred into 500-μl drops of M16, and cumulus cells were removed by treatment with hyaluronidase (300 μg/ml) for 3–5 min. Zona-intact eggs were washed in 1 500-μl drop and 4 100-μl drops of M16 and then transferred to a fresh 100-μl drop of M16 to which chymotrypsin (10 μg/ml) was added. Zonae pellucidae were removed by passing eggs through a narrow-bore Pasteur pipette. Zona-free eggs were quickly transferred into a 100-μl drop of M16 and washed through 4 more drops, then incubated in M16 at 37°C/5% CO_2 in air for 3–4 h during the sperm capacitation period. During this incubation and about 1 h before insemination, eggs were preloaded with 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride (Polysciences, Inc., Warrington, PA), 10 μg/ml, for 12–14 min and then washed through 4 100-μl drops of M16. The DAPI-stained eggs were returned to M16 until use in IVF assays.

Sperm were obtained by removing the cauda epididymis and vas deferens from mature males and cutting the tissues into a few pieces with surgical scissors in Whittingham's medium, 3% BSA. This mixture was incubated 15 min to allow sperm to swim out. Tissue pieces were removed, then sperm were diluted to approximately 5×10^5/ml and allowed to capacitate and acrosome react in Whittingham's medium, 3% BSA (37°C/5% CO_2) for ≥3 h. In some assays, undiluted sperm or ~5×10^6 sperm/ml were treated with inhibitor and then diluted further immediately before insemination.

In vitro fertilization assays. A variety of compounds were tested in IVF experiments to screen for an inhibitory effect on sperm–egg fusion. The following solvents with and without inhibitor were added to the insemination drops (maximum final concentration): 0.5% DMSO ± actinonin (20 μM); M2 ± captopril (100 μM); 0.02 M Tris–glycine, pH 8.0, ± α_2-macroglobulin (1000 units of activity); 1% DMSO ± E64 (15 μM); 1% DMSO ± Pefabloc SC (1 mM); 1% DMSO ± pepstatin (1.46 μM); 1–2% DMSO ± 4,7- or 1,10-phenanthroline (1 mM); M2 ± phosphoramidon (250 μM); 1% DMSO ± succ-Ala-Ala-Ala-AMC or succ-Ala-Ala-Phe-AMC (3 mM); and 1% DMSO ± Ro 31-9790 (200 μM). The above solvents (e.g., 1–2% DMSO) were found not to affect the gamete fusion assay. Test inhibitors or solvent controls were added to diluted sperm at the end of the capacitation and acrosome reaction period. Approximately 10 min after the addition of solvents ± samples,

zona-free eggs were added to the test and control sperm drops and incubated for 40 min.

In experiments testing the effects of 1,10-phenanthroline after it was "washed out" of IVF medium, zona-free eggs were treated with 1 mM 1,10-phenanthroline during the last 30 min of sperm capacitation. The eggs were then washed through 4 100-μl drops of M16 and added to diluted, capacitated sperm for fertilization (Fig. 1c). Alternatively, capacitated sperm ($\sim 2 \times 10^7$ or 5×10^6/ml) were incubated with 1 mM 1,10-phenanthroline for 30 min and then diluted 100-fold in fresh medium. Zona-free eggs were added to these washed sperm for fertilization (Fig. 1d). In other experiments, capacitated sperm ($\sim 5 \times 10^5$/ml) were co-incubated with zona-free eggs for 15 min and then 1 mM 1,10-phenanthroline was added to the insemination drop for the final 25 min of the incubation period (Fig. 1e).

In experiments testing inhibitory activities of purified TIMPs, TIMPs at various concentrations up to 1.2 μM were added to the test inseminations. Equivalent amounts of the buffer and salt present in the TIMP sample, but no TIMP, were added to the control inseminations.

In all IVF experiments, inseminated eggs were washed in 2 100-μl drops of M16 (the first contained ~0.5% paraformaldehyde) and transferred to microscope slides prepared with an elevated coverslip. Sperm binding and sperm–egg fusion were scored by light microscopy using a Zeiss Axiophot microscope, with a 10× or 20× objective lens. Binding was scored by counting sperm heads bound to eggs and then calculating the mean number of bound sperm per egg. Fusion was scored by counting sperm heads that were fluorescently labeled within the egg cytoplasm, due to transfer of DAPI stain from the DAPI-preloaded eggs. Two measures of fusion were determined in all assays: fertilization rate (FR, percentage of eggs fused with at least one sperm) and fertilization index (FI, mean number of fused sperm per egg). Data were compared using the Student *t* test. The IC_{50} for a protease inhibitor is defined as the inhibitor concentration that gives half of the maximal inhibition obtainable with that compound.

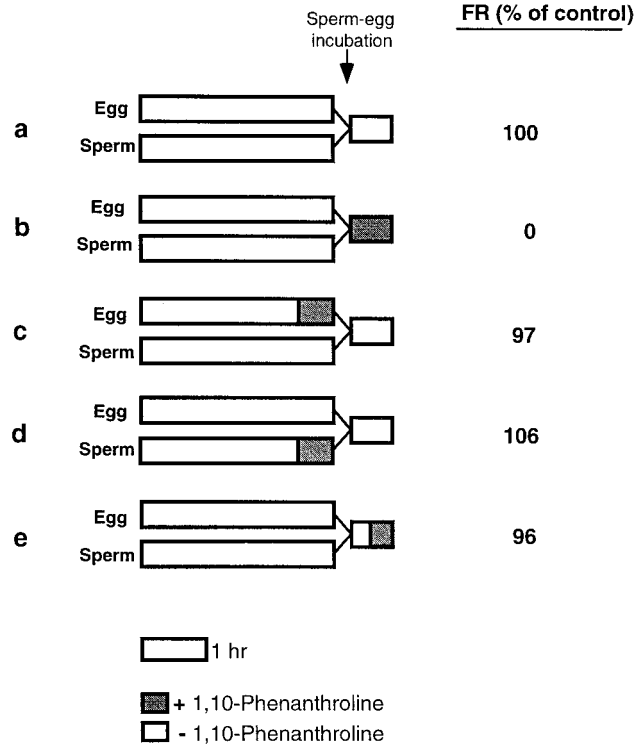

FIG. 1. Phenanthroline, a zinc chelator, inhibits fusion only when present during initial sperm–egg plasma membrane contact. In these assays, 1 mM 1,10-phenanthroline was used. The basic protocol is shown in (a): zona-free eggs were incubated in M16+0.3% BSA, while sperm were allowed to capacitate and acrosome react for ≥3 h. After capacitation and acrosome reaction, gametes were co-incubated for 40 min. In (b), 1,10-phenanthroline was present throughout the 40-min insemination period. In washout experiments, 1,10-phenanthroline was added either to eggs (c) or to sperm (d), during the last 30 min of sperm capacitation, then washed out as described under Materials and Methods. In (e), sperm–egg incubation was initiated for 15 min, then 1,10-phenanthroline was added to the insemination drop for the remaining 25 min of fertilization. FR is expressed as percentage of control (no phenanthroline) value. The total number of eggs tested was (a) 115, (b) 50, (c) 30, (d) 43, and (e) 28.

RESULTS

Metalloprotease Inhibitors Block Sperm–Egg Fusion

In order to determine if one or more proteases play a role in the process of mouse gamete fusion, we tested class-specific protease inhibitors and a synthetic protease substrate using *in vitro* adhesion and fusion assays. We have previously found that when mouse sperm are capacitated for ≥3 h with the protocol used here, spontaneous acrosome reaction reaches a maximal level, ~60% (Cho *et al.*, 1998; unpublished results). The current experiments were designed so that the capacitation period was ≥3 h to ensure that spontaneous acrosome reaction had reached a maximal level before a protease inhibitor was added (the basic protocol is shown in Figs. 1a and 1b). The inhibitors tested were chosen based on their ability to inhibit virtually all members of one specific protease class and have often been used to determine the mechanistic class of a newly identified protease (Beynon and Bond, 1986). Results from these experiments are summarized in Table 1. Pepstatin, E64, and Pefabloc SC are inhibitors of the aspartic, cysteine, and serine protease classes, respectively. These three inhibitors were tested at concentrations inhibitory in other systems (Beynon and Bond, 1986) and had no inhibitory effect on sperm–egg binding or fusion, although they did have an effect on sperm viability. Incubation of pepstatin and E64 with sperm and eggs caused about 50% of the sperm to die compared to control assays, yet the remaining sperm were viable and had normal motility. Despite the effects on sperm viability, enough sperm survived during the incubation period to bind to and fuse with eggs at levels equivalent to controls (Table 1). Similarly, we observed that after ~35 min of the sperm–egg incubation with Pefabloc SC, virtually all sperm had stopped moving. However, in the pres-

TABLE 1

Inhibitors of the Metalloprotease Class of Proteases Inhibit Sperm–Egg Fusion

Class inhibited	Inhibitor (concentration)[a]	IVF[b,c] controls FR	IVF controls FI	IVF controls No. sperm/egg	IVF inhibitors FR	IVF inhibitors FI	IVF inhibitors No. sperm/egg
All classes, not all proteases	α_2 Macroglobulin (1000 units)	79	1.5	8.3	87	1.6	10.3
Aspartic proteases	Pepstatin (1.5 μM)	94	1.1	2.0	87	1.1	5.6
Cysteine proteases	E64 (30 μM)	94	1.1	2.0	82	1.1	3.4
Serine proteases	Pefabloc SC (1 mM)	87	1.3	5.5	87	0.9	18.5
Metalloproteases	AAF (3 mM)	See Table 2			Inhibits		
	1,10-Phenanthroline (1 mM)	See Figs. 2 and 3			Inhibits		

[a] The maximum concentrations tested are given; these concentrations give strong inhibition in systems sensitive to the inhibitor.

[b] IVF assays, +/− the inhibitor, were used to assess inhibitory activity. Inhibitors were added to capacitated and acrosome-reacted sperm ($\sim 5 \times 10^5$ sperm/ml), then zona-free eggs were added to the sperm drops. After a 40-min incubation period, FR, FI, and No. sperm/egg were determined. The total number of eggs tested for each protease inhibitor and corresponding buffer control, respectively, were α_2 macroglobulin, 50, 48 (means, $n = 3$; P values for FR, FI, and No. sperm/egg compared to controls were ≥ 0.75); pepstatin, 15, 17; E64, 16, 17; Pefabloc SC, 15, 15. *Note.* Pepstatin, E64, and Pefabloc SC reduced sperm viability during sperm and egg incubation, yet caused no significant inhibition of the fertilization parameters, therefore these experiments were not repeated.

[c] The following abbreviations are used: AAF, succ-Ala-Ala-Phe-AMC; FI, fertilization index; FR, fertilization rate; IVF, *in vitro* fertilization; No. sperm/egg, number of sperm bound per egg.

ence of Pefabloc SC, sperm were bound to and fused with zona-free eggs at levels either higher than (No. sperm bound) or equal to (% eggs fused) the control levels (Table 1).

Inhibitors of the metalloprotease class were found to strongly inhibit sperm–egg fusion. The tripeptide substrate succ-Ala-Ala-Phe-AMC (Roe *et al.*, 1988) at relatively high concentration will inhibit metalloproteases by competing with endogenous substrates for protease binding (discussed by Beynon and Bond, 1986). We found that this peptide gave somewhat variable but substantial inhibition of sperm–egg fusion, while the control tripeptide succ-Ala-Ala-Ala-AMC, which is not a metalloprotease substrate, had little effect on fusion (Table 2). The variation observed was well within that observed in normal fusion assays. Neither of the two peptides tested decreased the number of sperm bound per egg. We also tested the zinc metalloprotease inhibitor 1,10-phenanthroline, a zinc chelator, and found that gamete fusion was inhibited (Table 1), while binding was either unaffected or slightly increased (see below). Incubation of sperm with 1,10-phenanthroline (up to 5 mM) had no effect on sperm motility or on the ability of sperm nuclei to be stained by DAPI (data not shown). Based on these findings, it appears that aspartic, cysteine, and serine proteases are not involved in gamete fusion, although they may play a role related to sperm viability. On the other hand, metalloproteases may be involved in the fusion process, in one or more steps after sperm bind to the egg plasma membrane.

A dose–response curve was determined for inhibition of sperm–egg fusion by 1,10-phenanthroline, using increasing concentrations of chelator added after capacitation and the acrosome reaction (see Fig. 1b for experimental design). As shown in Fig. 2, inhibition of sperm–egg fusion was concentration-dependent, with a virtually complete block at 500 μM and a complete block at 1 mM phenanthroline. In contrast, there was no decrease (and perhaps a small increase) in sperm–egg binding, which indicates that the inhibited metalloprotease(s) functions after plasma membrane binding (Fig. 2).

Additional experiments were done to determine if the inhibitor must be present during sperm–egg interactions in order to block gamete fusion. When eggs or sperm were incubated with phenanthroline for the last 30 min of the capacitation period, followed by washing (eggs) or dilution into fresh medium (sperm), sperm–egg fusion was not

TABLE 2

Effect of Synthetic Metalloprotease Peptide Substrate on Sperm–Egg Fusion

Experiment[a]	Peptide	Concentration (mM)	FR[b]	FI	No. sperm/egg
I	None	—	100	1.6	8.4
	AAA	3	90.0	1.4	7.6
	AAF	3	38.9	0.4	12.1
II	AAA	2	93.3	1.0	5.6
	AAF	2	6.3	0.1	9.3

[a] IVF assays were used to assess inhibitory activity. Inhibitors were added to capacitated and acrosome-reacted sperm, then zona-free eggs were added to the sperm drops. After a 40-min incubation period, FR, FI, and No. sperm/egg were determined. The total number of eggs tested were Expt. I, 21 (no peptide), 20 (AAA), and 18 (AAF); Expt. II, 15 (AAA) and 16 (AAF).

[b] The following abbreviations are used: AAA, succ-Ala-Ala-Ala-AMC; AAF, succ-Ala-Ala-Phe-AMC; FI, fertilization index; FR, fertilization rate; IVF, *in vitro* fertilization; No. sperm/egg, number of sperm bound per egg.

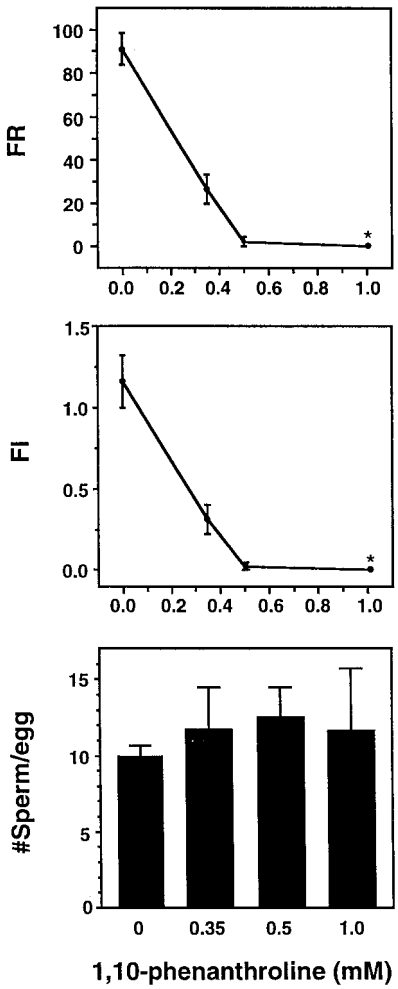

FIG. 2. The zinc chelator 1,10-phenanthroline blocks sperm–egg fusion without decreasing binding. In IVF assays, 1,10-phenanthroline was present throughout the 40-min insemination period. The values shown are the means ± SEM; $n = 3$. The P values for 0.35 and 0.5 mM 1,10-phenanthroline compared to no inhibitor are <0.003 and 0.0003 (FR) and < 0.01 and 0.002 (FI), respectively. There was no statistically significant difference in the mean number of sperm bound/egg compared to the control value. The * indicates that there were no fused nuclei observed at 1.0 mM 1,10-phenanthroline. The total numbers of eggs tested in each group were 69, 77, 77, and 72 (for 0, 0.35, 0.5, and 1.0 mM 1,10-phenanthroline, respectively).

inhibited in either case (Figs. 1c and 1d). These findings indicate that the metalloprotease activity is not irreversibly inhibited by exposure to phenanthroline.

Characterization of the kinetics of sperm–egg fusion in our assay shows that at 15 min of sperm–egg co-incubation the fertilization rate is 30–50% of the final fertilization rate obtained at 40 min of sperm–egg co-incubation (data not shown). Thus at 15 min, at least half the fertilizable eggs have not yet fused. When phenanthroline was added to insemination drops at 15 min after sperm–egg co-incubation began, there was no inhibition of sperm–egg fusion obtained at 40 min (Fig. 1e). This suggests that the metalloprotease acts during the initial 15-min co-incubation period of sperm and egg.

It is possible that a small hydrophobic compound such as phenanthroline may inhibit membrane fusion nonspecifically by intercalating into the plasma membrane and causing a change in the lipid organization. To test this possibility, a nonchelating structural isomer, 4,7-phenanthroline, was tested in gamete fusion assays concurrently with the zinc chelator, 1,10-phenanthroline. As shown in Fig. 3, 1,10-phenanthroline inhibited fusion in a dose-dependent manner, while 4,7-phenanthroline had no effect on sperm–egg fusion (P values for 4,7-phenanthroline relative to control for FR and FI: 0.427 and 0.313, respectively). In addition, 4,7-phenanthroline had no effect on gamete binding (data not shown). These findings suggest that the observed inhibition of fusion by 1,10-phenanthroline is due to its ability to act as a chelator.

Metalloproteases require metal ions, usually zinc, for catalytic activity. 1,10-Phenanthroline has a much higher stability constant for Zn^{2+} (2.5×10^6 M^{-1}) than for Ca^{2+} (3.2 M^{-1}) and thus is a potent zinc chelator and a weak calcium chelator. Consequently, 1,10-phenanthroline at 1 mM will inhibit zinc metalloproteases in the presence of up to 10 mM calcium (discussed in Beynon and Bond, 1986). Calcium in the range of 1.0–2.5 mM is optimal for mouse sperm–egg fusion (Evans *et al.*, 1995). The Ca^{2+} concentration (1.8 mM) in our IVF medium should not be significantly affected by 0.5–1.0 mM 1,10-phenanthroline which is sufficient to block fusion. Therefore, 1,10-phenanthroline presumably inhibits gamete fusion by removing zinc ions required for activity of a putative Zn^{2+}-dependent metalloprotease.

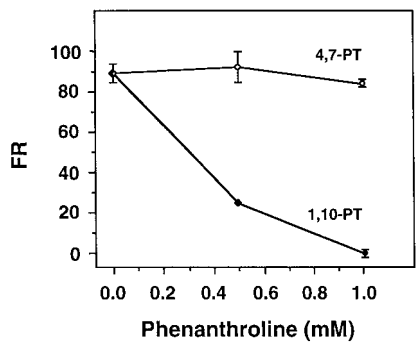

FIG. 3. A nonchelating phenanthroline isomer does not inhibit sperm–egg fusion. IVF assays, using both 4,7- and 1,10-phenanthroline at the indicated concentrations, were carried out as described for Figs. 1a and 1b. The mean FR is shown ± SEM; $n = 6$ for control (no addition) assays, $n = 2$ for 0.5 mM and $n = 4$ for 1 mM 4,7-phenanthroline. The P value for comparison of control and 1 mM 4,7-phenanthroline assays = 0.837.

TABLE 3
Effects of Family-Specific Metalloprotease Inhibitors on Sperm–Egg Fusion

Family inhibited	Inhibitor (concentration)[a]	IVF[b,c] controls FR	IVF controls FI	IVF controls No. sperm/egg	IVF inhibitors FR	IVF inhibitors FI	IVF inhibitors No. sperm/egg
Gluzincin class							
Aminopeptidase, ACE	Actinonin (20 μM)	88	1.4	9.5	89	1.4	10.6
ACE, Endopeptidase, and Thermolysin	Captopril (100 μM)	77	0.9	*	86	1.0	*
	Phosphoramidon (250 μM)	91	1.5	*	94	1.5	*
Metzincin class							
MMP	TIMP-1[d] (1.2 μM)	See Fig. 5			No inhibition		
MMP, ADAM	TIMP-3[d] (1.2 μM)	See Fig. 5			Inhibits		
MMP, ADAM	Ro 31-9790[e] ($\geq$25 μM)	See Fig. 4			Inhibits		

[a] The maximum concentrations tested are given; these concentrations give strong inhibition in systems sensitive to the inhibitor.
[b] IVF assays were used to assess inhibitory activity. Inhibitors were added to capacitated sperm, then zona-free eggs were added to sperm drops. After a 40-min incubation period, FR, FI, and No. sperm/egg were determined. The total number of eggs tested for each inhibitor and corresponding buffer control, respectively, were actinonin, 9, 8; captopril, 31, 31; phosphoramidon (concentrations from 50 to 250 μM were tested and none had an effect on fertilization), 83, 89. The * indicates that the number of sperm bound/egg was not counted for these experiments; however, no differences were observed in sperm binding between control and inhibitor groups by microscopic examination of the insemination drop.
[c] The following abbreviations are used: ACE, angiotensin converting enzyme; ADAM, a disintegrin and metalloprotease domain, adamalysin; FI, fertilization index; FR, fertilization rate; IVF, *in vitro* fertilization; MMP, matrix metalloprotease; No. sperm/egg, number of sperm bound per egg; TIMP, tissue inhibitor of metalloprotease.
[d] Generously provided by Dr. Gillian Murphy.
[e] Generously provided by Roche Discovery, Welwyn, England.

Assignment of the Metalloprotease(s) Involved in Sperm–Egg Fusion to a Specific Family of Zn^{2+}-Metalloproteases

A variety of more specific inhibitors of Zn^{2+}-metalloproteases was tested in fertilization assays *in vitro*, in an effort to narrow down the identified activity to one or a few families. Zinc metalloproteases have been divided into several classes based on the sequences in and surrounding their zinc binding sites (Hooper, 1994). The names of these classes are gluzincins, metzincins, inverzincins, and carboxypeptidases. Within each class, there are several distinct families. Inhibitors are available for specific families in the gluzincin class and metzincin class.

Inhibitors were tested at concentrations that yield strong inhibition in other systems; the results of this set of experiments are summarized in Table 3. Actinonin, an antibiotic and hydroxamic acid derivative, has been reported to be a strong inhibitor of the metalloexoproteases aminopeptidase M and leucine aminopeptidase (Umezawa *et al.*, 1985), members of the aminopeptidase family. Actinonin did not inhibit sperm–egg binding or plasma membrane fusion. Phosphoramidon and captopril were first described as potent inhibitors of the thermolysin family of metalloproteases (Komiyama *et al.*, 1975; Rasnick and Powers, 1978; Nishino and Powers, 1979). Phosphoramidon is also a very potent inhibitor of mammalian endopeptidase-24.11, as well as other metalloproteases not classified in the thermolysin family (Bond and Butler, 1987; Roques and Beaumont, 1990; Hooper, 1994; Beynon and Bond, 1986). Captopril is a strong inhibitor of the mammalian exoprotease angiotensin converting enzyme (ACE), of the ACE family of metalloproteases (Roques and Beaumont, 1990; Beynon and Bond, 1986). Since a testis-specific isoform of ACE appears to be present on the sperm plasma membrane (Langford *et al.*, 1993; Sibony *et al.*, 1994), ACE inhibitors were important to test. It has been well documented that phosphoramidon and captopril do not inhibit the matrixin (MMP) family (Bond and Butler, 1987; Gearing *et al.*, 1994; Beynon and Bond, 1986; Couet *et al.*, 1996) and at least one member of the adamalysin/reprolysin family of metalloproteases (Mohler *et al.*, 1994; Gearing *et al.*, 1994). Neither phosphoramidon nor captopril had an effect on sperm–egg binding or fusion (Table 3).

Inhibitors specific for the MMP family were tested in fusion assays to determine if this family may play a role in sperm–egg fusion (Table 3). The MMPs are mammalian proteases, such as the gelatinases, collagenases, stromelysins, and matrilysin, that play important roles in degradation and remodeling of the extracellular matrix (Woessner, 1991; Matrisian, 1992; Sato *et al.*, 1994; Brooks *et al.*, 1996; Couet *et al.*, 1996; Holmbeck *et al.*, 1999). The TIMPs specifically inhibit MMPs and thus are an integral component of the regulation of these metalloproteases *in vivo* (Apte *et al.*, 1995).

The hydroxamic acid derivative Ro 31-9790 (provided by Roche Discovery Welwyn, England), a broad-spectrum MMP inhibitor, significantly inhibited sperm–egg fusion. As illustrated in Fig. 4, the presence of Ro 31-9790 in the

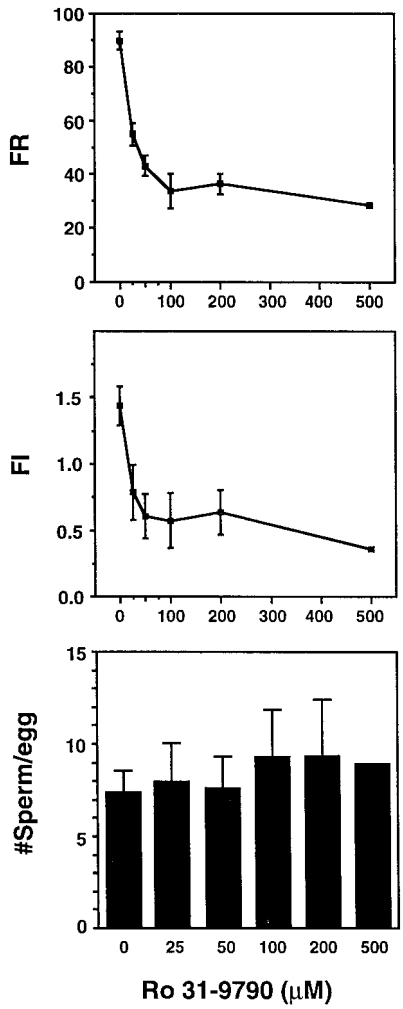

FIG. 4. The matrixin and adamalysin metalloprotease inhibitor Ro 31-9790 inhibits sperm–egg fusion without decreasing sperm–egg binding. IVF assays using increasing concentrations of Ro 31-9790 were used to assess inhibitory activity. The mean number of sperm bound per egg, FR, and FI are shown, ± SEM. For assays with 0, 25, 50, 100, 200, and 500 μM Ro 31-9790, n = 8, 4, 3, 4, 3, and 1, respectively. The P values for FR at all Ro 31-9790 concentrations compared to no inhibitor are $\leq$0.0001; P values for mean number of sperm bound/egg at all concentrations are not significant (ranging from 0.443 to 0.906). The total numbers of eggs tested in each group were 145, 82, 64, 84, 63, and 14 (for 0, 25, 50, 100, 200, and 500 μM Ro 31-9790, respectively).

insemination drop resulted in substantial, dose-dependent inhibition of fusion. Incubation of gametes with all concentrations of Ro 31-9790 tested caused a significant decrease in fertilization rate (for 25 to 200 μM, P values relative to controls for FR were $\leq$0.0001; Fig. 4). The IC_{50} for Ro 31-9790 inhibition of sperm–egg fusion was ~20 μM. None of the concentrations tested caused a reduction in the number of sperm bound per egg (Fig. 4).

Two members of the TIMP family of proteins, TIMP-1 and TIMP-3, were also tested in IVF assays. TIMP-1, which inhibits essentially all MMPs except membrane type-1 MMP (MT1-MMP; Will *et al.*, 1996) and MMP-19, did not inhibit sperm–egg binding or fusion (Fig. 5). However, TIMP-3, an inhibitor of MMPs including MT1-MMP (Will *et al.*, 1996), and the adamalysin TACE (Amour *et al.*, 1998), did strongly inhibit sperm–egg fusion (Fig. 5). The IC_{50} for inhibition of sperm–egg fusion by TIMP-3 was ~0.6 μM. Furthermore, TIMP-3 did not significantly affect the average number of sperm bound per egg in these assays.

DISCUSSION

In the present study, we investigated whether metalloprotease activity is important for fusion of gametes during mammalian fertilization. Our results suggest that one or more zinc metalloproteases must act after the sperm binds to the egg plasma membrane in order for sperm and egg to proceed to membrane fusion. We found that four distinct kinds of metalloprotease inhibitors block sperm–egg fusion without any effect on sperm–egg plasma membrane binding. The active inhibitors are the peptide substrate succ-Ala-Ala-Phe-AMC, the zinc chelator 1,10-phenanthroline, the hydroxamic acid derivative Ro 31-9790, and the endogenous MMP inhibitor TIMP-3. A substantial variety of other inhibitors, which block other protease classes (aspartic, cysteine, and serine proteases) or certain other families of zinc metalloproteases, do not affect gamete fusion.

We found that the zinc chelator 1,10-phenanthroline, a broad-spectrum inhibitor of zinc metalloproteases, can completely block mouse sperm–egg fusion. The inhibitory effect of 1,10-phenanthroline is reversible: if either gamete is incubated with the chelator and then washed free of it, gamete fusion is unaffected. Additionally, the inhibitor must act within a short time window: if 1,10-phenanthroline is added 15 min after sperm–egg co-incubation begins, it has no effect on fusion. These findings pinpoint a relatively brief time, the first 15 min of sperm and egg co-incubation, in which the zinc metalloprotease step(s) takes place.

Inhibitors of gluzincin families and of specific metzincin families did not inhibit gamete fusion. However, the compound Ro 31-9790, a hydroxamic acid derivative that is an inhibitor of various metzincins in the matrixin and adamalysin families, did inhibit sperm–egg fusion. The IC_{50} of Ro 31-9790 for inhibition of gamete fusion is ~20 μM. This is higher than the IC_{50} of Ro 31-9790 (2–3 μM) reported for inhibition of the proteolytic release of L-selectin from the cell surface, catalyzed by the adamalysin TACE (Preece *et al.*, 1996; Allport *et al.*, 1997; Peschon *et al.*, 1998). Similarly, TIMP-3 protein strongly inhibited sperm–egg fusion, while TIMP-1 protein had no effect on fusion in IVF assays. In another study, TIMP-3 was found to inhibit the protease activity of recombinant TACE, while TIMP-1 did not (Amour *et al.*, 1998). The IC_{50} for TIMP-3 inhibition of

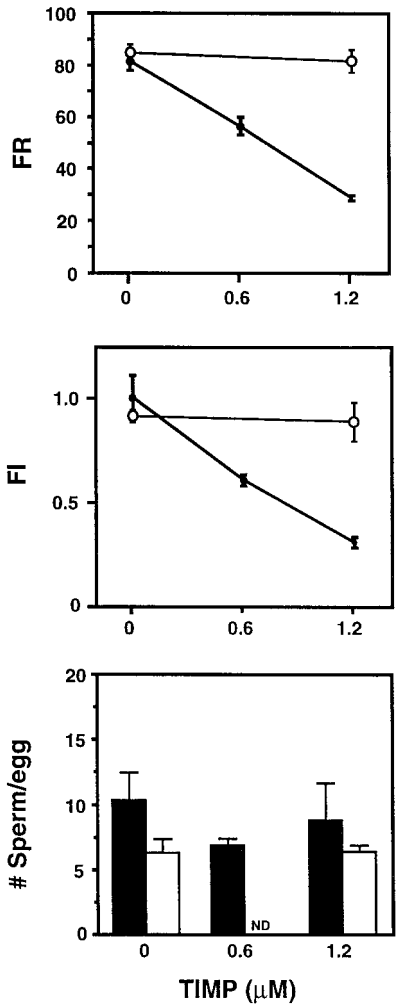

FIG. 5. TIMP-3 but not TIMP-1 inhibits sperm–egg fusion and does not decrease sperm–egg binding. TIMP-1 (open circles and open bars) and TIMP-3 (closed circles and closed bars) were tested in IVF assays as described. The FR, FI, and mean number of sperm bound/egg are given ± SEM. For TIMP-1 and corresponding control assays (no TIMP-1 added), $n = 3$, and for TIMP-3 and corresponding control assays (no TIMP-3 added), $n = 4$. There was no statistically significant difference in FR and FI for TIMP-1 compared to the appropriate control (no TIMP-1 added) nor for the mean number of sperm bound per egg for TIMP-1 and TIMP-3 compared to corresponding controls. The *P* values for FR and FI for TIMP-3 compared to no inhibitor added are 0.004 and 0.02 at 0.6 μM TIMP-3 and <0.0001 and 0.0006 at 1.2 μM TIMP-3, respectively. The total numbers of eggs tested in assays using TIMP-1 were 66 and 71 (for 0 and 1.2 μM), and the total numbers of eggs tested in assays using TIMP-3 were 116, 70, and 107 (for 0, 0.6, and 1.2 μM, respectively). ND, not determined.

fusion in our study was ~0.6 μM, similar to that reported for TIMP-3 inhibition in other systems. For example, the IC_{50} for TIMP-3 inhibition of shedding of L-selectin from leukocytes was reported to be 0.3–0.4 μM (Borland *et al.*, 1999).

The maximum level of inhibition by either TIMP-3 (at the highest level tested, 1.2 μM) or Ro 31-9790 (at 100 μM) was ~60%, while 1,10-phenanthroline was able to completely block sperm–egg fusion. Based on these findings, we suggest that there may be two zinc metalloproteases involved in sperm–egg fusion. One may be a member of the matrixin or adamalysin family and highly sensitive to TIMP-3 and Ro 31-9790 inhibition, while the second may be from a different family, insensitive to these inhibitors but sensitive to 1,10-phenanthroline. Alternatively, there may be a single gamete metalloprotease related to but (in terms of TIMP-3 and Ro 31-9790 sensitivity) different from other members of the matrixin and adamalysin families. The putative gamete metalloprotease might be structurally different and have a rate of activity in the presence of saturating TIMP-3 and Ro 31-9790 which is ~40% that of the uninhibited enzyme.

The metalloprotease activity studied here is clearly different from that studied previously by Meizel and colleagues. They posited a role for a metalloprotease in an earlier step in mammalian fertilization based on work using human sperm and hamster eggs (Diaz-Perez *et al.*, 1988; Diaz-Perez and Meizel, 1992). In their studies, sperm were incubated during the entire capacitation period with phosphoramidon (which has no effect on mouse gamete fusion with our experimental design), a nonspecific metal ion chelator (diethylenetriaminepentacetic acid), or a synthetic metalloprotease substrate (carbobenoxy-L-phenylalanine). Sperm were then washed and used in IVF assays. This protocol with these three inhibitors resulted in a 50–60% decrease in the average number of sperm that fused with eggs (fertilization index). Meizel and co-workers concluded that a metalloprotease activity was important during the acrosome reaction and presumably metalloprotease inhibition during the acrosome reaction indirectly affected later steps leading to sperm–egg fusion.

Our results with 1,10-phenanthroline and the synthetic metalloprotease substrate are strikingly similar to those found in the study of sea urchin gamete fusion (Roe *et al.*, 1988). Lennarz and colleagues found both the peptide succ-Ala-Ala-Phe-AMC and 1,10-phenanthroline had no effect on binding of acrosome-reacted sperm to the egg plasma membrane but strongly blocked subsequent membrane fusion. More specific synthetic metalloprotease inhibitors (like Ro 31-9790) or natural inhibitor proteins such as the TIMPs were not available at the time of this previous study. Results from the present study suggest the possibility that there is a metalloprotease, or at least a similar proteolytic process, that is conserved between invertebrate and mammalian gamete fusion.

Sensitivity to Ro 31-9790 and to TIMP-3 suggests that the inhibited gamete metalloprotease could be related to the matrixin or adamalysin families. Most metalloproteases of the matrixin family are secreted enzymes, although the recently identified subset of membrane-type matrix metalloproteases (MT-MMPs) contains a transmembrane domain (Sato *et al.*, 1994; Strongin *et al.*, 1995; Will *et al.*, 1996).

The gamete metalloprotease identified in our study, presumably a membrane protein since the activity was inhibited at the surface of gametes, may be comparable to the MT-MMP group of matrix metalloproteases. This is supported by the findings that TIMP-3, which inhibits various MMPs including the MT-MMPs, inhibited sperm–egg fusion, while TIMP-1, an inhibitor of all MMPs except MT1-MMP and MMP-19, did not affect sperm–egg fusion.

A subset of the adamalysins, the ADAMs, is a family containing plasma membrane anchored metalloproteases (Primakoff and Myles, 2000). ADAMs have an important role in the proteolytic release (shedding) of cell surface ectodomains from other plasma membrane proteins (Mohler *et al.*, 1994; Arribas *et al.*, 1996; Preece *et al.*, 1996; Middlehoven *et al.*, 1997; Hooper *et al.*, 1997; Peschon *et al.*, 1998; Qi *et al.*, 1999). Frequently the released, soluble ectodomain is the active form of a cytokine (e.g., tumor necrosis factor α) or a growth factor (e.g., transforming growth factor β). It is possible that the gamete surface metalloprotease sheds another gamete membrane protein ectodomain that is active in a soluble form and functions in the membrane fusion process. Alternatively, the shedding of ectodomains could be a prerequisite to very close approach of the lipid bilayers, which is necessary for fusion.

From our data we cannot conclude if the putative MMP and/or ADAM-related metalloprotease(s) active in fusion is located on the sperm surface or egg surface or perhaps both. To our knowledge, there is no report of a metalloprotease on the egg surface. The ACE metalloprotease is present on the sperm surface (Langford *et al.*, 1993; Sibony *et al.*, 1994) but ACE inhibitors do not affect gamete fusion (Table 3). Currently eight ADAM proteases that are testis-specific have been identified and at least some, and perhaps all, of these are expressed on the surface of mature sperm (Primakoff and Myles, 2000; our unpublished results). Certain ADAM proteases are sensitive to Ro 31-9790 (Allport *et al.*, 1997; Middlehoven *et al.*, 1997; Peschon *et al.*, 1998) and TIMP-3 (Amour *et al.*, 1998). Furthermore, it is possible that MMPs or TIMPs may be expressed by sperm (Nothnick *et al.*, 1998; our unpublished results). To answer directly if a particular ADAM or MMP, or any other sperm surface metalloprotease, is the identified gamete metalloprotease with a role in gamete fusion will require further investigation. Other outstanding questions include what are the substrates of the gamete surface metalloprotease, what factors regulate its activity, and what is its specific contribution to the membrane fusion process?

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